

Rec'd PCT/PTO 18 OCT 2004

FLUORESCENCE TECHNIQUE FOR ON-LINE MONITORING OF
STATE OF HYDROGEN-PRODUCING MICROORGANISMS

5 CONTRACTUAL ORIGIN OF THE INVENTION:

The United States Government has rights in this invention pursuant to Contract No. DE-AC36-99GO10337 between the United States Department of Energy and the Midwest Research Institute.

BACKGROUND OF THE INVENTION

10 1. Field of the Invention

The invention relates to providing a non-destructive, external or remote, on-line way to monitor the physiological state of stressed (inclusive of nutrient stress and sulfur-deprivation stress) oxygenic photosynthetic microorganisms that have a hydrogenase *in situ* with regard to their ability to produce H₂ gas without having to rely on electrodes or
15 other sensors placed in direct contact with the culture medium.

2. Description of the Prior Art.

The ability of eukaryotic microalgae to produce H₂ after a dark, anaerobic induction period was discovered about 60 years ago; however, the enzyme catalyzing this activity, a reversible Fe-hydrogenase, is inactivated by trace amounts of O₂ that is co-evolved
20 during photosynthesis. Thus, light-dependent H₂ production by green algae, including *Chlamydomonas reinhardtii*, is observed for only a very short period of time after anaerobic induction. Several years ago, Wykoff et al.¹ found that incubating cells deprived of inorganic sulfur for up to 120 h, resulted in the progressive reduction of photosynthetic capacity due to the inactivation of photosystem II (PSII) O₂-evolution
25 activity by up to 95%.

Subsequent work [Melis et al.² and Ghirardi et al.³] investigated this effect more thoroughly and showed that after 1-2 days of adaptation to sulfur-deprived conditions, *C. reinhardtii* cells could produce volumetric amounts of H₂ gas for a few days under continuous illumination. The key observation made was that, when the rate of
30 photosynthetic O₂ evolution declined below the rate of cell respiration, the culture could

1. 1998. Plant Physiol. V. 117. P. 129-139.

2. 2000. Plant Physiol. V. 122. P. 127-136.

3. 2000 a Trends Biotechnol. V. 18. P. 506-511

make itself anaerobic. Under these conditions, the hydrogenase enzyme was activated and/or induced in the light, and the onset of H₂ production followed after several hours. It has been demonstrated that the process involved the sequential transition through the following five physiological phases: O₂-evolution, O₂- consumption, anaerobic adaptation, H₂-production, and termination phases. After this occurred, the culture could be regenerated by adding sulfate, prior to another round of H₂ production. Nevertheless, the actual mechanisms involved in and the time-course for the decrease in PSII activity in sulfur-deprived cells during cell transition to the anaerobic state as well as the role of PSII in H₂ are not well established.

10 U.S. Patent 5,372,784 discloses measurement of bacterial CO₂ production in an isolated fluorophore by monitoring an absorbance-regulated change of fluorescence. The fluorophore is positioned to intersect the transmission light path, and indirectly monitors absorbance or changes in the absorbance of a chromophore encapsulated or isolated by a gas permeable polymetric matrix.

15 A biological treatment plant controlled by fluorescence sensors is disclosed in U.S. Patent 5,700,370. The method of using these fluorescence sensors comprises:
monitoring the microbiological activity of the biological system and/or fluctuations thereof by on-line measurement of fluorescent emission and/or variations therein for at least one characteristic biogenic fluorophore present in the mixed culture of
20 microorganisms in the system when irradiated with light and controlling one or several parameters of the process by using results from the measurement as measured variable(s) in an on-line automatization system.

U.S. Patent 5,981,958 discloses a method and apparatus for detecting pathological and physiological change in plants. This invention provides an imaging
25 fluorometer comprising a source of electromagnetic radiation, optical components to direct the radiation, excitation and emission filters, and an imaging-device. Radiation from the radiation source is used to excite fluorescence from a dark-adapted sample containing photosynthetic components. This fluorescence is collected by the imaging device as a function of time and position within the sample. Excitation and emission
30 filters limit the intensity and wavelengths of radiation incident on the sample and imaging device, respectively.

A method and material for determining relative abundance of microorganisms in fixed populations is disclosed in U.S. Patent 6,107,033. The method entails:

- 1) providing a set of labeled *in situ* hybridization cluster oligonucleotide probes;
- 2) hybridization of said probes with a sample of the mixed population, and
- 5 3) quantitative analysis of the number of labeled microorganisms.

German Patent, DE 19930865 discloses methods for detecting phytoplankton content of natural water samples by chlorophyll fluorescence measurements. The measurements allow distinguishment among different algae groups on the basis of chlorophyll fluorescence measurements using a measuring beam in combination with strong pulses of light with different intensities, frequencies, phases, and wavelengths.

The system of this patent includes:

a first light source for producing a weak measuring light for exciting fluorescence in a selected portion of the sample volume;

a second light source for illuminating the remaining volume of the sample with a light intense enough to induce phototaxis (e.g., by dinoflagellates);

means for detecting and recording the time-dependent changes in light in the sample volume and the portion of the sample undergoing measurement; and

means for comparing the changes to known values.

The time-dependent changes in fluorescence exhibit relative amplitudes and time constants, which are characteristic of certain dinoflagellate types.

Karukstis et al., in Photochemistry and Photobiology (1992) Vol. 55. No. 1 pp. 125-132 disclose "*ALTERNATIVE MEASURES OF PHOTOSYSTEM II ELECTRON TRANSFER INHIBITION IN ANTHRAQUINONE-TREATED CHLOROPLASTS*" that compare electron direct transport assays with an indirect fluorescence assay for basic research studies.

An *ON-LINE MONITORING OF CHLOROPHYLL FLUORESCENCE TO ASSESS THE EXTENT OF PHOTOINHIBITION OF PHOTOSYNTHESIS INDUCED BY OXYGEN CONCENTRATION AND LOW TEMPERATURE AND ITS EFFECT ON THE PRODUCTIVITY OF OUTDOOR CULTURES OF SPIRULINA PLATENSIS (CYANOBACTERIA)* is disclosed by Torzilla et al. in J. Phycol. Vol. 34 (1998) pp. 504-510.

Polle et al. Biohydrogen II, (workshop) (2001) Meeting date 1999. pp. 111-130 disclose *MAXIMIZING PHOTOSYNTHETIC EFFICIENCIES AND HYDROGEN PRODUCTION IN MICROALGA CULTURES* by developing algal mutants with smaller antenna sizes.

5 There is a need for providing a technique for on-line monitoring of the state of hydrogen-producing microorganisms under aerobic and anaerobic conditions inside a closed photobioreactor system from an external vantage point to gain information about the state of the culture without using electrodes inserted directly into the culture medium, and thereby preclude the possibility of a source of (a) culture contamination
10 and the need to sterilize electrodes, and (b) gas, including oxygen and hydrogen, leaks, to produce a non-destructive, remote sensing procedure.

SUMMARY OF THE INVENTION

One object of the present invention is to provide a process for determining the main factor(s) in regulating H₂ production under sulfur depletion of oxygenic
15 photosynthetic microorganisms that have a hydrogenase.

Another object of the present invention is to provide a process for determining the main factor(s) in regulating H₂ production under sulfur depletion of oxygenic photosynthetic microorganisms that have a hydrogenase, by monitoring the culture from an external vantage point.

20 A further object of the present invention is to provide a process for determining the main factor(s) in regulating H₂ production under sulfur depletion of oxygenic photosynthetic microorganisms that have a hydrogenase, by monitoring the culture from an external vantage point, without using electrodes or other type of sensor inserted directly into the culture medium.

25 A yet further object of the present invention is to provide a process for determining the main factor(s) in regulating H₂ production under sulfur depletion of oxygenic photosynthetic microorganisms that have a hydrogenase, by monitoring the culture free from a source of culture contamination or gas leakage, that remotely senses the culture in a manner that is non-destructive to the sample.

30 In general, the invention process is accomplished by monitoring the physiological state of sulfur-deprived algal cultures that progress through the

physiological phases of: O₂ production, O₂ consumption, anaerobic adaptation, H₂ production, and termination, to ascertain a number of significant and well-defined fluorescence parameter changes during and between these phases; namely, to the effect that, at the exact time that sulfur-stressed algae become anaerobic, the following three
 5 *in situ* fluorescence parameters change dramatically and are used to determine when and if a culture in a photobioreactor goes anaerobic:

- F_t (the steady-state level of chlorophyll (Chl) fluorescence in light-adapted cells) increases abruptly;
- 10 • F_m' (the maximal saturating light-pulse induced fluorescence level in light-adapted cells) decreases abruptly; and
- $\Delta F/F_m' = (F_m' - F_t)/F_m'$ (the calculated photochemical activity of photosystem II [PSII] under steady-state illumination) decreases precipitously and abruptly.

The *in situ* fluorescence method for on-line monitoring of the state of sulfur-
 15 deprived algal culture to ascertain the culture's ability to produce H₂ under sulfur depletion entails:

- a) providing a sample of sulfur-deprived algal culture containing photosynthetic components;
- b) illuminating the sample with artificial or natural illumination;
- 20 c) determining the onset of H₂ photoproduction by measuring the percentage of H₂ in a produced gas phase at multiple times to ascertain the point immediately after the anerobiosis subsequent to the physiological phases of O₂ production and O₂ consumption sequence to obtain data regarding H₂ as a function of time; and
- 25 d) determining any abrupt change in the following three *in situ* fluorescence parameters:
 - i) an abrupt increase in F_t (the steady-state level of chlorophyll fluorescence in light adapted cells);
 - ii) an abrupt decrease in F_m' (the maximal saturating light
 30 induced fluorescence level in light adapted cells); and
 - iii) a precipitous and abrupt decrease in $\Delta F/F_m' = (F_m' - F_t)/F_m'$

(the calculated photochemical activity of photosystem II (PSII)) that signal the full reduction of the plastoquinone pool between PSII and PSI, which indicates the start of anaerobic conditions that in turn induces the synthesis of the hydrogenase enzyme required for subsequent H₂ production, and thereafter slowing down of the abrupt decrease and partial recovery of $\Delta F/F_m'$ signal at least partial oxidation of the plastoquinone pool as the main factor to regulate H₂ production under sulfur depletion.

BRIEF DESCRIPTION OF THE DRAWINGS FIGURES

FIG. 1 is a graph showing the effect of 10 μ M DCMU on the rate of H₂ photoproduction by cultures of sulfur-depleted *C. reinhardtii* cells, where the control cells are open circles and the DCMU-treated cells, are closed articles. DCMU was added to treated cells at the arrow. Time zero in this case represents the time after H₂ photoproduction starts.

FIG. 2 is a graph of a time course of physiological parameters and H₂ production in *C. reinhardtii* cells during incubation under sulfur-deprived conditions. More particularly, FIG. 2A shows *in situ* fluorescence parameters F_t (open squares), $\Delta F/F_m'$ (solid circles), and $\Delta F_m'$ (open triangles) and chlorophyll concentrations (Chl; solid triangles) as a function of time, and FIG. 2B shows dissolved oxygen (pO₂, crosses), redox potential (E_h (stars) and H₂ gas collected in an inverted graduate cylinder (solid squares). Incubation in sulfur-deprived medium started at 0 h.

FIG. 3 is a graph showing *in situ* fluorescence parameters F_t (open squares), $\Delta F/F_m'$ (solid circles), and F_m' (open triangles), as well as pO₂ (crosses), E_h (stars) and H₂ content in the gas phase of culture vessel (solid squares) in illuminated, sulfur-deprived, *C. reinhardtii* during the transition of the algae from aerobic to anaerobic conditions. Incubation in sulfur-deprived medium started at 0 h.

FIG. 4 is a graph showing changes in chlorophyll fluorescence parameters recorded in a dark-adapted algal sample, which was removed anaerobically from a

culture vessel 22 hours after the beginning of H_2 production. After 18 minutes of dark adaptation, the algal sample was aerated (arrow). F_0 (open squares), F_m (open triangles), and F_v/F_m (solid circles) were monitored periodically as a function of time after removal from the culture vessel.

5 FIG. 5 is a graph showing chlorophyll fluorescence induction curves in control algae at the start of sulfur deprivation (A) and in cells removed from a culture vessel anaerobically after 22 hours of H_2 production (B, C, D). (B) Fluorescence kinetics were recorded while the cells were illuminated with saturating light after a 10-minute period of dark adaptation. (C) Same as (B) except far red
10 light ($\lambda \sim 735$ nm) was turned on for one second prior to the measurement. (D) Same as (B) except that the sample was aerated just prior to measurement. The F_0 level occurs at time zero.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENT OF INVENTION

Chlamydomonas reinhardtii strain Dangeard C137+ was grown in Tris-acetate-phosphate (TAP) medium (pH, 7.0) under continuous cool-white fluorescent light. Late-log cells were pelleted three times by centrifugation and re-suspended in the same medium, from which inorganic sulfure (TAP-S) was omitted. The cells were incubated under continuous fluorescent illumination ($200\text{--}250\ \mu\text{E m}^{-2}\ \text{s}^{-1}$ PAR) in cylindrical reactor vessels (600 ml) with a rotating glass rod and Teflon impellers for mixing. A magnetic bar was attached at the bottom of the rod to couple it to an external stirring motor, and the rate of mixing was 60-80 rpm. The vessels were sealed using a silicon stopper with an outlet tube inserted through it for gas collection. The gas produced by the cells was collected in an inverted graduated glass cylinder by water displacement.

To determine the precise moment of H_2 -photoproduction onset, we measured the percentage of H_2 contained in the gas phase (~ 7 ml) on the top of the vessel immediately after anaerobiosis by gas chromatography. The dissolved O_2 and redox potential of the medium were also measured. Chlorophyll concentrations were then obtained spectrophotometrically in 95% ethanol cell extracts. The capacity for respiratory O_2 consumption was measured with a Clark electrode at 25°C under aerobic conditions (17% or $170\ \mu\text{M O}_2$) after removing the samples from the culture vessel and equilibrating them with air for 5 minutes.

Chlorophyll fluorescence measurements were obtained with a portable PAM-2000 fluorometer using the weak modulated pulse-probe (Pulse-Amplitude-Modulation [PAM]) method. *In situ* measurements of fluorescence yield ($\lambda > 710\ \text{nm}$) were done with an optical fiber probe affixed closely onto the surface of the illuminated glass reactor vessel. The measurements were recorded every 15 minutes over the total period of sulfur deprivation. A 0.8 s saturating actinic excitation pulse ($\lambda < 710\text{nm}$, $1200\ \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-2}$ PAR) from an 8V/20 W halogen lamp was applied on top of weak modulated probe flashes (3 μs pulses from a 655 nm light-emitting diode (LED) at frequencies of 600 Hz or 20kHz.) for FIGS 2-4, whereupon the following parameters were recorded: (a) F_0 , the fluorescence yield of dark-adapted cells measured only in the

presence of weak probe flashes; (b) F_m , the fluorescence yield of the same cells, following the application of the saturating actinic pulse; (c) F_t , the fluorescence yield of the cells, exposed to the ambient, steady-state illumination from fluorescence lamps or natural lighting in the reactor vessels, measured with the weak probe flashes; and (d) F_m' , the fluorescence yield of illuminated cells, as in (c), following application of the saturating actinic pulse. The efficiency of photochemical conversion of absorbed light energy (the photochemical activity) in PSII was calculated both after dark adaptation, where $F_v/F_m = (F_m - F_0)/F_m$, and under steady-state actinic light illumination, where $\Delta F/F_m' = (F_m - F_t)/F_m'$. The kinetics of fluorescence induction was also measured with the PAM-2000 fluorometer. Samples were removed anaerobically from the reactor vessel and placed into a transparent plastic cuvette hermetically sealed with a stopper. Following a 10-minute dark, anaerobic adaptation period, the samples were illuminated with weak modulated probe flashes as above and actinic light (655 nm, $250 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR from a LED array for 2s), and the kinetics of fluorescence induction were measured at $\lambda > 710 \text{ nm}$ (FIG. 5).

As mentioned above the sulfur-deprivation process involves O_2 -production, O_2 -consumption, anaerobic-adaptation, H_2 -production and termination phases. These phases proceed over different periods of time, depending on the residual sulfur concentration (at the time of sulfur removal), chlorophyll and biomass content in the culture, pH of the culture medium, and other factors. This is why the beginning of the anaerobic phase is observed at different times after sulfur-deprivation in different experiments. The observed changes in cell parameters during the sulfur-deprivation process (excluding FIGS. 2 and 3) are expressed relative to the indicated physiological stage of the cultures.

One of the most critical questions about the mechanism of H_2 photoproduction in sulfur-deprived systems is the identification of the source of the electrons for gas production. The electrons could come from storage products or from water (i.e., electrons from water are transported from PSII to PSI), with subsequent reduction of ferredoxin and hydrogenase.

FIG. 1 shows the addition of DCMU (a potent inhibitor of electron flow at the level of Q_B [the secondary PSII quinone acceptor] on the reducing side of PSII) to a

culture, after H_2 gas production has started, abruptly decreases the rate of gas production by 80%. This demonstrates that most of the electrons from H_2 production are coming from water and is consistent with the fact that large amounts of H_2 are only produced in the light. The direct involvement of PSII-catalyzed water oxidation activity by sulfur-deprived cells was further investigated using PAM fluorescence techniques.

During the O_2 -production and consumption phases of sulfur deprivation (the first 15 hours in the experiment shown in FIG. 2A), the Chl concentration was set at $11 \mu\text{g/ml}$ at the beginning of the experiment, and the *in situ*-monitored fluorescence parameter, F_m' , did not change significantly. However, since the fluorescence parameter, F_t , slowly increased during this same period, the photochemical activity of PSII ($\Delta F/F_m'$), declined from 0.57 in the middle of the O_2 -production phase to 0.44 at the end of the O_2 -consumption phase (FIG. 2A). The observed changes in F_m' , F_t and $\Delta F/F_m'$ reflect the existence of long-lived inactive states apparently generated in the Q_B -non-reducing centers of PSII. In fact, if samples of the algal suspension were removed aerobically from the culture vessels at the end of the O_2 -consumption phase and dark-adapted for different periods of time, the fluorescence parameters, F_0 and F_m , and the fluorescence induction curves did not change significantly during a 40-minute incubation period (data not shown). This suggests that the Q_B -non-reducing state of PSII is long-lived in the dark once it is established.

The results are consistent qualitatively with previous observations showing that the capacity for O_2 production by PSII, the amount of light-reducible Q_A^- , (reduced form of the primary PSII quinone acceptor) and the rate of electron transport from PSII and PSI decrease during sulfur deprivation. However, it is noteworthy to point out that the experiments of Wykoff et al. and Melis et al. were done by removing samples from a photobioreactor and assaying for O_2 evolution under aerobic conditions, whereas our results are from *in situ* measurements.

FIG. 2A also shows that, in this experiment at 15 and 16 h after the start of sulfur deprivation, there was a rapid (< 5 min) reduction of the *in situ*-measured photochemical yield of PSII ($\Delta F/F_m'$) from 0.4 to 0.1, which reflects a rapid down-regulation of PSII photochemical activity at this time. The loss of photochemical

activity was due both to a rapid reduction in F_m' and a rapid increase in F_t . It is important to note that in FIG. 3 (an expanded view of data from FIGS. 2A and B) the observed rapid reduction of PSII photochemical activity ($\Delta F/F_m'$) began at the exact time that the O_2 concentration in the culture suspension reached zero. The redox potential of the culture medium also started to decline around this time (FIGS 2B and 3) but not nearly so fast as the O_2 concentration and PSII photochemical activity. At about one hour of anaerobiosis, the PSII photochemical activity dropped to a minimum value (0.05). After that point, it gradually increased over the next 2 hours and reached a maximum value of 0.085 (FIGS. 2A and 3). No additional large changes were observed over 20 more hours of anaerobiosis and H_2 production. If at this point (21 h after the beginning of anaerobiosis), an algal sample was removed anaerobically from the reactor vessel and placed into an anaerobic cuvette for 18 minutes in the dark, the PSII activity (F_v/F_m) of the dark-adapted cells increased rapidly from 0.07 to 0.14 when the cell suspension was subsequently aerated (FIG. 4). Following the aeration, the F_v/F_m ratio increased slowly up to a value of 0.22 after an additional hour of aerobic incubation. This clearly demonstrates that the rapid *in situ* anaerobic inactivation of PSII photochemical activity is partially reversible by O_2 in contrast to the slower aerobic inactivation of PS II observed during the O_2 consumption stage of sulfur deprivation.

Volumetric measurements of H_2 gas accumulation, such as those presented in FIG. 2B, do not give the exact time of the start of H_2 photoproduction. To determine the exact onset of H_2 production, we measured the concentration of H_2 in the gas phase of the reactor vessel (FIG. 3). Trace quantities of H_2 (0.002%) were detected as early as 10 minutes after the onset anaerobiosis. This is reflected at the point where the decrease in The $\Delta F/F_m'$ slows dramatically. The rate of H_2 accumulation in the gas phase increased from 0.04 μl of H_2 per minute within the first 10 minutes of anaerobiosis up to 1.0 μl per minute at the end of the second hour of anaerobiosis. This was accomplished by a moderate increase in the $\Delta F/F_m'$ value. Gas accumulation in the inverted graduate cylinder started after about 4 h of anaerobiosis (FIG. 2B), and the rate of H_2 accumulation was constant until at least 22 h after the beginning of anaerobiosis. These results suggest that the algae actually start to produce H_2 very

shortly after the establishment of anaerobiosis. Apparently it takes a couple of hours to saturate the algal suspension with H₂ (the solubility of H₂ in water is 17 ml per l at 30°C) and then build up enough gas pressure to displace the water in the gas-collection system. We show earlier that, under different initial conditions of cell cultivation
5 and/or less accurate measuring conditions, the *in situ* measured photochemical yield of PSII drops to zero after the rapid decrease in photochemical yield. In the current study the observed recovery or up-regulation of PSII activity under anaerobic conditions correlates with the onset of H₂ production. This correlation confirms that PSII, and consequently residual water-splitting function, is the main source of electrons of H₂
10 production under these conditions.

As to the actual mechanisms responsible for the observed *in situ* changes in PSII activity in sulfur-deprived cultures, the initial slow inactivation of PSII capacity is believed to be due to the decreased ability of the cells to replace photodamaged D1 protein, thus leading to the accumulation of Q_B-non-reducing PSII centers that cannot
15 produce O₂. These changes result in the establishment of anaerobic conditions in the reactor vessel in the light.

In addition to the slow inactivation of PSII capacity, we demonstrate that a new, rapid down-regulation of PSII photochemical activity occurs just as the culture becomes anaerobic (FIGS. 2 and 3). This rapid down-regulation is not accompanied by
20 a proportional loss of PSII capacity as measured by the capability of PSII to accumulate Q_A- or to evolve O₂ when the cells are exposed to aerobic conditions [FIG. 4]. It is believed that the rapid *in situ* PSII down-regulation is a response of the algae to the over-reduction of the plastoquinone (PQ) pool, which must accompany the establishment of anaerobiosis. Since there is no O₂ to reoxidize the PQ pool under
25 anaerobic conditions and since PSI cannot further dispose of electrons from the pool (the hydrogenase is not active yet and there is little rubisco to utilize photosynthetically-generated electrons), PSII activity is reversibly down-regulated. A similar mechanism, but slower rate has been used to explain the irreversible loss of PSII activity in eukaryotic algae following exposure to high temperatures or when
30 deprived of nutrients other than sulfur.

The evidence presented in FIG. 5 supports our suggestion that rapid down-

regulation of PSII is induced by over-reduction of the PQ pool under anaerobic conditions. It is known that the rise from F_0 to the F_i level on the fluorescence induction curve corresponds to the portion of PSII centers that are unable to reduce Q_B . A control, aerobic algal sample, removed from the aerobic culture vessel at the start of sulfur deprivation (see FIG. 2), was examined first. In this sample (FIG. 5A), the F_i peak is followed by a slower rise of fluorescence to the ∞ -maximum level, and this reflects the reduction of the PQ pool. In contrast, fluorescence-induction curves recorded anaerobically from anaerobic, dark-adapted cells, 22 h after the start of anaerobic phase (FIG. 5B), had a pronounced F_i peak but completely lacked a slow rise phase. The lack of the slow rise indicates that the PQ pool cannot be further reduced under these conditions. Accordingly, the F_i peak disappeared if the algal suspension was pre-illuminated with far red light (FIG. 5C) or aerated (FIG. 5D) prior to recording the fluorescence kinetics. In the former case, the PQ pool was oxidized by PSI, which preferentially absorbs far red light, and in the latter it was oxidized by O_2 itself. The loss of the distinct F_i peak occurs because oxidation of a small fraction of the PQ pool under these conditions reoxidizes Q_B and this lowers the F_i level to that of the steady-state value on this time scale. Under anaerobic conditions, reduction of the PQ pool occurs by residual photosynthetic electron transfer and/or by the transfer of reductants from anaerobic substrate degradation in the chloroplast stroma to the PQ pool through the NAD(P)H-PQ oxidoreductase. In fact, the existence of such reductant transfer in our samples is confirmed by incomplete (80%) suppression of H_2 production after the addition of DCMU (FIG. 1).

The consumption of photosynthetically produced O_2 during the H_2 -production phase is essentially for maintaining the culture medium anaerobic for the operation of the hydrogenase. Oxygen consumption in *C. reinhardtii* is catalyzed by three main oxidases: (a) the chloroplast PQ-oxidase; (b) the mitochondrial cytochrome c-oxidase; and (c) the mitochondrial alternative oxidase. The existence of the respiratory electron transport process, chlororespiration, occurring in the chloroplasts of green algae explains the effect of respiratory inhibitors on chlorophyll fluorescence transients. The chlororespiratory chain allows the transfer of reductants (NADPH), generated by substrate degradation in the chloroplast stroma, to the PQ pool via the NAD(P)H-PQ

oxidoreductase.

The PQ pool is further oxidized by dissolved O_2 through the action of PQ-oxidase. In the mitochondria, NADH and $FADH_2$ generated by the Krebs Cycle serve as the source of electrons for a membrane-bound electron transport chain with O_2 as the final electron acceptor. The cytochrome oxidase transduces the production of 3 ATP molecules per pair of electrons transported. The alternative oxidase, on the other hand, is actually active under conditions that require less ATP/NADH since its operation generates only 2 ATP/pair of electrons. In both cases, O_2 is the terminal electron acceptor. The mitochondrial cytochrome oxidase in *C. reinhardtii* is inhibited by KCN, carbon monoxide, and sodium azide, while the alternative oxidase is specifically affected by salicylic hydroxamic acid (SHAM). The putative chloroplast PQ oxidase, on the other hand, is not inhibited by SHAM and only slightly affected by KCN. By measuring the influence of two inhibitors, KCN and SHAM, on the rates of dark respiration, we tried to estimate the relative contributions of each of the three oxidases at different times after sulfur deprivation. The actual contributions of the three oxidases to the scavenging O_2 *in situ* depend on many factors such as their relative K_M 's for O_2 and the rate of diffusion of O_2 from the chloroplast to the mitochondria. Thus, the inhibitor studies that follow may not directly reflect the actual situation in the photobioreactor, but they do indicate that changes in the relative contribution of the three oxidases occur over the course of sulfure deprivation.

Table 1 shows that sulfur-deprivation gradually inhibits the rates of dark respiration in *C. reinhardtii* over a 100-h incubation period.

TABLE I

Phase of Sulfur depletion	Rate of respiration ($\mu\text{moles O}_2/\text{mg Chl}^{-1} \times \text{h}^{-1}$)	%KCN inhibition (% CO)	%SHAM inhibition (% AO)	Putative % PQ-oxidase (% CR)
Start of sulfur depletion	30	60	25	15
Start of anaerobic phase	28	16	0	84
Start of H ₂ production phase	22	36	13	51
During H ₂ production	18	8	38	54
End of H ₂ production	6	17	100	0

Contributions to the mitochondrial cytochrome oxidase [CO], mitochondrial
 5 alternative oxidase [AO], and chlororespiration [CR] to the total respiration rate of *C. reinhardtii* cells are also shown during sulfur-deprivation. Respiration rates were measured with cells taken from the bioreactor at different times after sulfur depletion, equilibrated with air and incubated with the inhibitors for 5 min. Rates were measured when the O₂ concentration in the medium was 17% O₂. Final concentrations of KCN
 10 and SHAM were 5 mM and 4 mM, respectively.

At the start of sulfur deprivation, the cytochrome oxidase and the alternative
 oxidase contributed, respectively, about 60 and 25% to the overall respiration capacity of the cells measured in the presence of 17% (170 μM) O₂. The remainder (about
 15 15%) appears due to the activity of the chloroplast PQ-oxidase (Table I, last column),; however, it should be noted that the specificity of the inhibitors is not 100%. At the start of the anaerobic phase, the activity of the two mitochondrial oxidases decreased substantially to 16 and 0% of the total respiration capacity, respectively. This indicates a shift from mitochondrial respiration to chlororespiration. The shift towards
 chlororespiration was coupled to the shut-down of electron transport from PSII (FIGS.
 20 2 and 3) caused by the over-reduction of the PQ pool, and the complete removal of O₂ from the sulfur-deprived culture medium. Once H₂ evolution started and accompanying

up-regulation of PSII began, photosynthetically-generated O_2 appeared to be consumed equally by the mitochondrial (49%) and chloroplast (51%) oxidases (see Table I). During the H_2 -production phase, the contribution of the chlororespiratory PQ oxidase remained unchanged. However, over the same period of time, the contribution of the
5 mitochondrial cytochrome oxidase declined dramatically while the alternative oxidase increased. At the end of the H_2 -production phase, the mitochondrial respiratory activity was due mostly to the alternative oxidase. Thus, the inhibitor studies in Table I support the active role of chlororespiration in maintaining the anaerobic conditions in the chloroplast required for H_2 photoproduction, and they are also consistent with the idea
10 that the alternative oxidase is expressed preferentially under stress conditions. This differential activity has also been observed in phosphate- or nitrogen-depleted *C. reinhardtii* and tobacco cells.

From FIGS. 2 and 3, it can be seen that the sharp down-regulation of PSII photochemical activity is reflected in an increase in F_t , which is the result of a sharp
15 increase in the number of active PSII centers that are unable to reduce Q_B . Decreases in F_m' are due to increases in non-photochemical quenching of PSII, and one of the known mechanisms responsible for non-photochemical quenching is the generation of a ΔpH gradient across the photosynthetic membrane. These fluorescence studies show that the sharp drop in F_m' at the time that O_2 concentration in the culture reaches zero
20 occurs at approximately the same time that the PQ pool becomes over-reduced. Since the oxidized PQ pool is the source of reductant for chlororespiration, the inhibitor studies (Table I) appear to indicate that the chlororespiratory pathway begins to make a major contribution to O_2 removal from the culture at about the same time. It therefore appears that the rapid decrease in PSII photochemical is also due to the enhancement of
25 chlororespiration, accompanied by the generation of a gradient. The increase in ΔpH appears to be the main functional mechanism of non-photochemical quenching under the conditions responsible for the observed drop in F_m' .

The observed up-regulation of PSII activity that follows the establishment of anaerobiosis in the culture vessel (FIGS. 2B and 3) appears to be the result of induction
30 of reversible Fe-hydrogenase enzyme activity, which catalyzes ferredoxin-dependent reduction of protons and releases H_2 . In fact, the hydrogenase provides a sink for

electrons generated either by light-catalyzed water oxidation or by metabolic oxidation of endogenous substrates, and its activity results in the partial oxidation of the intermediate photosynthetic carriers, including the PQ pool. Hence under anaerobic conditions, H₂ production by sulfur-deprived cells allows the system to retain a fraction
5 of the PSII reaction centers in a photochemically active state. The O₂ evolved under these conditions, as indicated, appear to be consumed in part by chlororespiration or by storage-product degradation linked to mitochondrial respiratory processes that utilize both the cytochrome oxidase and the alternative oxidase as the final electron acceptors.

In the context of the inventive process, it is important to note that, if only the
10 redox potential is measured without measuring the dissolved O₂ concentration, it is not possible to know exactly when the system goes anaerobic; however, the invention process measures the O₂ concentration in the culture at the same time that the fluorescence is measured to ascertain the exact time the system goes anaerobic (required for H₂ production). More specifically, the exact time the system goes
15 anaerobic is when the fluorescence (F/F_m') starts its abrupt drop, and this is the point where PSII activity is down-regulated.

In summarization, it is seen that changes of PSII activity in *C. reinhardtii* cells deprived of inorganic sulfur are characterized by complicated dynamics during the course of cell adaptation to the nutrient stress. We therefore conclude that (a) the redox
20 state of the PQ pool is the primary factor in regulating the activity of any remaining PSII water-splitting capacity by controlling the PSII photochemical activity in the algae during all phases of sulfur deprivation and (b) most of the electrons used directly for H₂ gas production come from water. Thus, the redox state of the PQ pool depends on the relationship between the rates of photosynthesis, chlororespiration, respiration, and
25 H₂ production.